EFFECT OF THE AGE CROSS-LINK BREAKER ALAGEBRIUM ON ANTERIOR SEGMENT PHYSIOLOGY, MORPHOLOGY, AND OCULAR AGE AND RAGE

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ABSTRACT

Purpose: To determine the effects of the advanced glycation end product (AGE) cross-link breaker alagebrium on intraocular pressure (IOP), accommodation (ACC), outflow facility (OF), anterior segment morphology, and ocular AGE and receptors for AGE (RAGE) in older rhesus monkeys.

Methods: Six rhesus monkeys (aged 19 to 20 years) received 3 or 4 intracameral and intravitreal (final concentration, 1 mM) injections of alagebrium to one eye over 2.5 to 3 weeks and vehicle to the opposite eye. ACC and OF responses to intramuscular or intravenous pilocarpine were measured at baseline and at 1 to 2 weeks and 2, 4, and 6 months postinjection. IOP was measured prior to all injections, ACC, and OF measurements. Monkeys were euthanized 3 to 6 months after the last injection, the eyes were enucleated, and anterior and posterior segments were examined by electron microscopy or immunohistochemistry.

Results: No significant differences were found in ACC or IOP at any time point after alagebrium treatment. Baseline OF was higher ($37.0 \pm 6.0\%$; $P \leq .005$) in alagebrium-treated vs control eyes at 6 months postinjection. In 3 monkeys, alagebrium-treated eyes, compared to control eyes, showed greater focal plaque formation, similar to that seen in primary open-angle glaucoma, in the juxtacanalicular meshwork/inner wall of Schlemm's canal. No changes in anterior segment AGE or RAGE were detectable. However, some areas of the retina and optic nerve head exhibited decreased AGE and increased RAGE immunostaining.

Conclusions: Intraocular injection of AGE cross-link breakers is an unlikely approach for glaucoma therapy. However, it may generate a model for further study of glaucomatous-like plaque formation. Immunohistochemical changes in the posterior segment in response to alagebrium warrant further functional studies.

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INTRODUCTION

Evidence suggests that the formation and accumulation of advanced glycation end products (AGEs) in connective tissue and extracellular matrix components account largely for the increase in collagen cross-linking that accompanies normal aging and that occurs at an accelerated rate in diabetes. AGEs accumulation in various tissues is associated with a number of age-related chronic diseases, including atherosclerosis, diabetes, arthritis, and neurodegenerative diseases.[1,2]

Structural matrix proteins such as collagen and elastin play an integral role in the maintenance of tissue elasticity and are prime targets for AGE cross-linking. One of the consequences of AGE cross-linking of collagen is decreased susceptibility to proteolytic and chemical degradation. The decreased proteolytic turnover results in an increased accumulation and continued AGE-derived cross-linking of collagen, leading to the loss of flexibility. Results of numerous studies indicate that myocardial stiffening is associated with an increase in the concentration and glycation of the collagen matrix.[3]

AGEs can also modulate cellular function through binding to the receptor of AGE (RAGE).[4] Binding of AGEs to RAGE can induce the release of profibrotic cytokines, such as TGF-β and proinflammatory cytokines, such as TNF-α and IL-6. Cell activation in response to AGE-modified proteins has also been associated with increased expression of extracellular matrix proteins, vascular adhesion molecules, and growth factors. RAGE-mediated events result not only in cell activation and proliferation, chemotaxis, and angiogenesis, but also in the generation of reactive oxygen species and apoptotic cell death.[5-9]

The effects of the AGE cross-link breaker phenyl thiazolium bromide (PTB) on vascular hypertrophy in streptozotocin-diabetic rats have been reported.[10] Intraperitoneal administration of PTB resulted in reduction of AGE accumulation on blood vessels and attenuated the diabetes-induced mesenteric vascular hypertrophy.[11] Subsequently, more stable and potent PTB analogs were developed. The first of these, ALT-711 (now known as alagebrium), was found to break AGE-derived cross-links in vivo when tested by oral administration to streptozotocin-diabetic rats after 7 weeks of diabetes.[10] The diabetes-induced increase of large artery stiffness was reversed as a result of 1 to 3 weeks of alagebrium treatment to rats.[12] Subsequent studies in dogs, monkeys, and humans have shown positive effects on several end points of vascular and cardiac stiffness.[1,2,13]

Alagebrium is the first orally active cross-link breaker being investigated for the treatment of diastolic and systolic heart failure. Currently in phase 2 clinical trials, alagebrium is designed to break glucose cross-links that develop as a result of both aging and diabetes and improve ventricular and arterial compliance and function.[14]

The eye is susceptible to AGE formation on account of its high oxidative and photo-oxidative stress environment and high ascorbic acid concentration. Various ocular tissues undergo structural and biochemical changes that have been associated with aging. Age-related increases in collagens have been reported in the trabecular meshwork[15,16] and lamina cribrosa.[17] AGEs may contribute to the reduced compliance in the lamina cribrosa of the optic nerve, which could increase the susceptibility of axons to damage from elevated intraocular pressure (IOP) in glaucoma.[17,18]

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Primary open-angle glaucoma (POAG) is a disease of aging and is a leading cause of blindness in the United States, especially for the elderly.[19] The exact cause of POAG is uncertain but in most instances likely involves the impairment of aqueous humor outflow over time.[20] Morphologic studies in aging and glaucoma eyes have shown an increase in accumulation of extracellular material in both the trabecular meshwork and ciliary muscle and a loss of trabecular meshwork cells, which contribute to this reduction in outflow facility and a consequent increase in IOP (reviewed in Gabelt and Kaufman[21]). This could result in part from decreased elasticity of the outflow pathway structures. Ultimately, IOP increases, often leading to damage of the optic nerve. Currently, the only therapeutic approach to treating POAG or ocular hypertension to prevent further vision loss is to lower the IOP, most commonly by pharmacologic intervention.[22,23]

Age can affect the outflow pathways and the efficacy of antiglaucoma therapy. Baseline outflow facility and uveoscleral outflow decrease with age in both humans and rhesus monkeys.[24-27] Ciliary muscle response to pharmacologic and central stimulation declines in aging live monkeys.[28,29] Most of the age-related decrease in responsiveness to cholinergic stimulation is thought to be due to stiffened posterior attachments of the ciliary muscle, restricting its forward movement.[30,31] Much remains to be learned about the physiology and responses to pharmacologic stimulation of the outflow pathways in the aging primate anterior segment and about their relationship to age-related morphologic changes.[31,32]

Presbyopia, the age-related loss in the ability to focus, is the most common ocular affliction in the world, affecting essentially 100% of the population over the age of 50. Changes in lenticular deformability alone can account for presbyopia. However, age-related posterior restrictive changes in the ciliary muscle can further limit accommodation and might do so even if the crystalline lens were replaced by a well-functioning artificial accommodating intraocular lens.[33] The same might be true of the anterior and posterior zonule. This loss in elasticity might be due in part to the formation of AGEs.

In theory, AGE cross-link-breaking compounds, such as alagebrium, could prove beneficial for ocular age-related disorders such as age-related macular degeneration, glaucoma, and presbyopia, as they have for age-related changes in the cardiovascular and other systems. By "loosening" or making more flexible the posterior attachments to the ciliary muscle, alagebrium may enhance the muscle's response to normal parasympathetic neuronal stimulations and to cholinergic agents such as pilocarpine that exert their IOP-lowering effect by contracting the ciliary muscle. Such contraction deforms the trabecular meshwork, thereby enhancing outflow of aqueous humor (outflow facility) from the eye.[34]

We hypothesized that alagebrium may break the cross-links formed by AGEs in the eye, thereby improving elasticity. In this study, we determined in monkeys whether the cross-link-breaker compound alagebrium can alter IOP, improve outflow facility via the conventional outflow pathways, and enhance the ability to focus in response to a pharmacologic stimulus. Postmortem eyes were then examined for morphologic changes and changes in AGEs and RAGEs. These studies were conducted in older, nonhuman primates that presumably have ocular age-related changes resulting from the formation of AGEs.

METHODS

All animal experiments were conducted in accordance with the University of Wisconsin Institutional Animal Care and Use Committee and National Institutes of Health guidelines and with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

ANIMALS, ANESTHESIA

Six female rhesus monkeys, 19 years old or older, in good health and free of any ocular abnormalities, were studied. Anesthesia was was with intramuscular. ketamine HCl (10mg/kg, supplemented with 5mg/kg; Phoenix Pharmaceuticals, St. Joseph, MO or Fort Dodge Animal Health, Fort Dodge, IA) for IOP and ketamine (10mg/kg, supplemented with 5mg/kg) + acepromazine (1mg/kg, Phoenix Pharmaceuticals) for refraction studies. For outflow facility measurements, animals were also given intravenous sodium pentobarbital (15 mg/kg, supplemented with 5 to 10 mg/kg; Ovation Pharmaceuticals, Deerfield, Illinois). At least 2 weeks prior to commencing the studies, both eyes of each monkey were surgically iridectomized[35] to facilitate refraction measurements. Prior to each procedure, eyes were examined by slit-lamp biomicroscopy. The absence of biomicroscopic cells and flare was confirmed prior to each stage of the study.

TRANSCORNEAL AND INTRAVITREAL INJECTIONS

Each ketamine-anesthetized monkey was placed supine in a head holder with the eyes directed upward. The eyes were cleansed with a betadine 5% solution (Alcon, Fort Worth, Texas) and balanced salt solution rinse (Alcon, Fort Worth, Texas). The corneas were anesthetized with one drop of topical proparacaine 0.5% (Alcaine; Alcon, Fort Worth, Texas). Alagebrium (Synvista Therapeutics, Montvale, New Jersey) was administered both intracamerally and intravitreally to treated eyes to a final concentration in each compartment of approximately 1 mM; Bárány's mock aqueous humor[36] was injected into the opposite eye. For intracameral injections, 43.68 μg alagebrium was dissolved in 14 μL Bárány's mock aqueous humor for each treated eye. The solutions were injected using a micrometer syringe connected to polyethylene tubing fitted with a 30-gauge needle. Using microscopic visualization, the needle was threaded through the cornea for several millimeters, and then the tip was angled into the anterior chamber, taking care not to touch the iris. The needle was left in place for 1 to 2 minutes after injection of the solutions to allow for hysteresis caused by the tensile properties of the tubing to subside. No fluid leakage was observed during needle withdrawal.

Intravitreal injections of 780 μg alagebrium dissolved in 25 μL Bárány's mock aqueous humor for each treated eye were given approximately 5 to 10 minutes later. For intravitreal injections, a 30-gauge needle, assembled as described above, was inserted through temporal pars plana 4 mm from the limbus to a depth of 8 mm into the mid vitreous and the alagebrium or vehicle delivered to the

corresponding eyes as above. Topical antibiotic ointment was applied following the injections. Each monkey was scheduled to receive 4 injections spaced 3 to 4 days apart if biomicroscopic examinations were normal. Table 1 shows a summary of the procedures and timing for each monkey. Intraocular pressure, accommodation, and outflow facility responses were determined at baseline and at approximately 2 to 3 weeks and 2, 4, and 6 months after the final alagebrium injection. Two of the monkeys were treated with a second round of 4 injections of alagebrium or vehicle, as above, ~6 months after the first round. Accommodation and outflow facility responses were again measured at 1 to 2 weeks and 2 months postinjection.

INTRAOCULAR PRESSURE, REFRACTION

Intraocular pressure was measured with a minified Goldmann applanation tonometer.[37] Refraction was measured using Hartinger coincidence refractometry (Carl Zeiss Meditech, Jena, Gemany). Accommodation was determined in response to intramuscular pilocarpine HCl (Sigma, 1.5 mg/kg in 3 mL 0.1% citrate buffer, pH 4.5) infused over a 10-minute period after pretreatment with 0.01 mg/kg intramuscular atropine sulfate (Abraxis Pharmaceuticals, Schaumberg, Illinois) to protect against systemic effects.[34] Refraction measurements were recorded every 5 minutes until a plateau was reached, usually 45 to 60 minutes from the start of the infusion. In some cases, if the accommodation response was less than 5 diopters, a second dose of pilocarpine was given and the measurements were repeated.

OUTFLOW FACILITY

Outflow facility was determined by two-level constant pressure perfusion of the anterior chamber with Bárány's perfusand. Animals were pretreated with 0.05 mg/kg intramuscular atropine. Following single-needle cannulation, a plano contact lens was placed on each cornea to maintain clarity for refraction measurements. Baseline outflow facility values were collected for 45 to 60 minutes. Monkeys were then given an intravenous injection of 1.5 mg/kg pilocarpine HCL in 3 mL citrate buffer over 10 minutes. Outflow facility was measured for another 60 minutes. Refraction was measured approximately every 10 minutes. Monkeys received continuous intravenous infusion of lactated Ringer solution (3 to 10 mL/kg/hr), and body temperature was maintained on water-circulated heating pads. There was at least a 4- to 6-week recovery period after outflow facility experiments before any additional studies were conducted. Outflow facility response was determined at baseline and at approximately 2 to 3 weeks and 2, 4, and 6 months after the final alagebrium injection.

DATA ANALYSIS

Data are expressed as the mean ± standard error of the mean. Significance was determined by the two-tailed paired *t* test for ratios compared to 1.0 or differences compared to 0.0.

TISSUE COLLECTION

After anesthesia was induced with intramuscular ketamine followed by pentobarbital plus Euthasol (Virbac Animal Health, Fort Worth, Texas) (morphology) or pentobarbital alone (immunohistochemistry), the monkeys were perfused through the heart with 750 mL phosphate-buffered saline (PBS) followed by 1 L of paraformaldehyde 4% followed by another 250 mL PBS. Eyes sent to Dr Lütjen-Drecoll in Germany were bisected into nasal and temporal halves with the optic nerve entirely in the nasal half. The nasal half was placed in Ito's fixative[38]; the temporal half was placed in paraformaldehyde 1%. The required permits were obtained from the Fish and Wildlife Service prior to shipping the tissues. For immunohistochemistry, eyes were bisected at the equator, immersed in paraformaldehyde 4% overnight, then transferred to PBS for shipment to Dr Tezel in Kentucky.

MORPHOLOGY

Fixed specimens from 3 pairs of eyes were cut into 1- to 2-mm wedges encompassing cornea, trabecular meshwork, iris, and ciliary body posteriorly to the ora serrata and were embedded in Epon. Semithin sections (1 µm) and ultrathin sections (60 nm) were cut using a Reichert Ultramicrotome (Vienna, Austria). Semithin sections were stained with toluidine blue, and ultrathin sections were counterstained with lead citrate and uranyl acetate.

IMMUNOHISTOCHEMISTRY

To determine the extent and cellular localization of AGE and RAGE in monkey eyes, immunoperoxidase labeling was performed in 3 pairs of eyes as previously described.[18] Briefly, all eyes were processed for 5-µm paraffin-embedded sagittal tissue sections. Histologic sections were deparaffinized, rehydrated, and pretreated with hydrogen peroxide, 3%, in methanol to decrease endogenous peroxidase activity. Following washing with PBS solution containing 0.1% bovine serum albumin, slides were incubated with 20% inactivated serum (Chemicon International Inc, Temecula, California) for 30 minutes at room temperature to block background staining. Slides were then incubated with a monoclonal mouse antibody against AGEs (1:100; Cosmo Bio Co, Tokyo, Japan) or a polyclonal goat antibody against RAGE (1:100; R&D Systems, Minneapolis, Minnesota) for 16 hours at 4°C. After washing, slides were incubated with the biotinylated anti-mouse or anti-goat IgGs (1:400; Chemicon International Inc) for 1 hour at room temperature, and then with ABC solution (Vector Laboratories, Burlingame, California) for 1 hour at room temperature. Following several washes, color was developed by incubation with 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St Louis, Missouri) as cosubstrate for 5 to 7 minutes. Slides were then counterstained with hematoxylin. The primary antibody was eliminated from the incubation medium, or serum (Sigma-Aldrich) was used to replace the primary antibody to serve as the negative control. After washing and

TABLE 1. SUMMARY OF SEQUENCE AND TIMING OF PROCEDURES FOR INDIVIDUAL MONKEYS

MONKEY NO.	AM67	AN02	AN07	AM78	AN55	AN63
DOB	10/2/85	6/16/86	6/24/86	5/7/86	4/22/87	5/5/87
BL SLE/IOP	5/18/05	5/18/05	5/18/05	8/8/06	8/8/06	8/8/06
Iridectomy	7/29/05	7/29/05	7/29/05	9/11/06	9/11/06	9/11/06
BL Acc	10/27/05	10/27/05	11/1/05	2/19/07	6/8/07	6/8/07
BL OF	11/10/05	11/10/05	11/11/05	2/27/07	6/28/07	6/28/07
Injection 1	2/3/06	4/10/06	4/10/06	4/4/07	9/4/07	9/4/07
SLE/IOP/Inj 2	2/7/06	4/21/06	4/21/06	4/10/07	9/6/07	9/6/07
SLE/IOP/Inj 3	2/13/06	4/26/06	4/26/06	4/13/07	9/13/07	9/13/07
SLE/IOP/Inj 4	2/20/06	5/5/06	5/5/06	4/20/07	Recurrent conjunctivitis	9/28/07
SLE/IOP/Acc	2/27/06	5/9/06	5/9/06	4/27/07		10/10/07
(1-2 wk)	7d/1wk	4d/1wk	4d/1wk	7d/1wk		12d/2wk
SLE/IOP/OF	3/13/06	5/18/06	5/18/06	5/3/07		10/23/07
(2-3 wk)	21d/3wk	13d/2wk	13d/2wk	13d/2wk		23d/3wk
SLE/IOP/Acc	4/10/06	6/20/06	6/20/06			
(4-8 wk)	49d/7wk/2mo	46d/6.5wk/2mo	46d/6.5wk/2mo			
SLE/IOP/OF	4/19/06	6/28/06	6/28/06			
(4-8 wk)	58d/8wk/2mo	54d/7.5wk/2mo	54d/7.5wk/2mo			
SLE/IOP/Acc				6/29/07	11/28/07	12/14/07
(9-12 wk)				70d/10wk/2.5mo	76d/10wk/2.5mo	77d/11wk/2.5mo
SLE/IOP/OF				7/13/07	12/10/07	
(9-12 wk)				84d/12wk/3mo	88d/12wk/3mo	
SLE/IOP/Acc	6/20/06	8/24/06	8/24/06	9/5/07		1/30/08
(16-20 wk)	106d/15wk/4mo	116d/16wk/4mo	116d/16wk/4mo	138d/20wk/5mo		124d/18wk/4mo
SLE/IOP/OF		9/14/06	9/14/06	9/11/07		2/12/08
(16-20 wk)		132d/18wk/4mo	132d/18wk/4mo	144d/20wk/5mo		137d/19wk/4mo
SLE/IOP/Acc	8/10/06				2/8/08	
(21-24 wk)	167d/23wk/5mo				148d/21wk/5mo	
SLE/IOP/OF	8/17/06				2/13/08	
(21-24 wk)	174d/24wk/6mo				153d/22wk/5mo	
SLE/IOP/Acc		10/31/06	10/31/06		3/25/08	4/1/08
(25-28 wk)		179d/25wk/6mo	179d/25wk/6mo		193d/27wk/6mo	185d/26wk/6mo
SLE/IOP/OF		11/9/06	11/9/06		4/2/08	4/8/08
(25-28 wk)		188d/26wk/6mo	188d/26wk/6mo		201d/28wk/6mo	192d/27wk/6mo
SLE/IOP/Acc				11/28/07		
(29-32 wk)				222d/32wk/8mo		
SLE/IOP/OF				12/10/07		
(29-32 wk)				234d/33wk/8mo		
SLE/IOP/Inj 5		12/4/06	12/4/06			
SLE/IOP/Inj 6		12/11/06	12/11/06			
SLE/IOP/Inj 7		12/18/06	12/18/06			
SLE/IOP/Inj 8		1/3/07	1/3/07			
SLE/IOP/Acc		1/11/07	1/11/07			
(1-2 wk)		8d/1wk	8d/1wk			
SLE/IOP/OF		1/18/07	1/18/07			
(2-3 wk)		15d/2wk	15d/2wk			
SLE/IOP/Acc		2/28/07	2/28/07			
(8 wk)		56d/8wk/2mo	56d/8wk/2mo			
SLE/IOP/OF		3/7/07	3/7/07			
(9 wk)		63d/9wk/2mo	63d/9wk/2mo			
Sac	11/2/06	4/12/07	4/12/07	1/31/08	8/8/08	8/8/08
Sac Method	Deep iso, perf 4%para+ PBS	Nembutal, perf 4%para+PBS	Nembutal, perf 4%para+PBS	Nembutal, perf 4%para+PBS	Nembutal, perf 4%para+PBS	Nembutal, perf 4%para+PBS
EM	Y	Y	Y			
Immunohist				Y	Y	Y

Acc, accommodation; BL, baseline; DOB, date of birth; EM, electron microscopy; Immunohist, immunohistochemistry; inj, injection; IOP, intraocular pressure; OF, outflow facility; para, paraformaldehyde; PBS, phosphate-buffered saline; perf, perfusion; Sac, sacrifice; SLE, slit-lamp examination; Y, yes.

mounting, slides were examined using a phase-contrast microscope, and images were recorded by digital photomicrography (Carl Zeiss Inc, Thornwood, New York). For each procedure, at least 10 histologic sections from each eye were utilized after masking the slides for the status of the monkey eyes.

RESULTS

INTRAOCULAR PRESSURE

Prior to the first injection, baseline IOP was 19.7 ± 0.7 in eyes to be treated with alagebrium and 19.8 ± 0.6 in eyes to be treated with vehicle (n = 6). The IOP (Figure 1) was not significantly different at any other time after injection of alagebrium or vehicle when comparing alagebrium-treated vs vehicle-treated eyes to their respective preinjection baselines except for 2 time points, which were likely due to chance, during the course of the 83 paired measurements that were done.

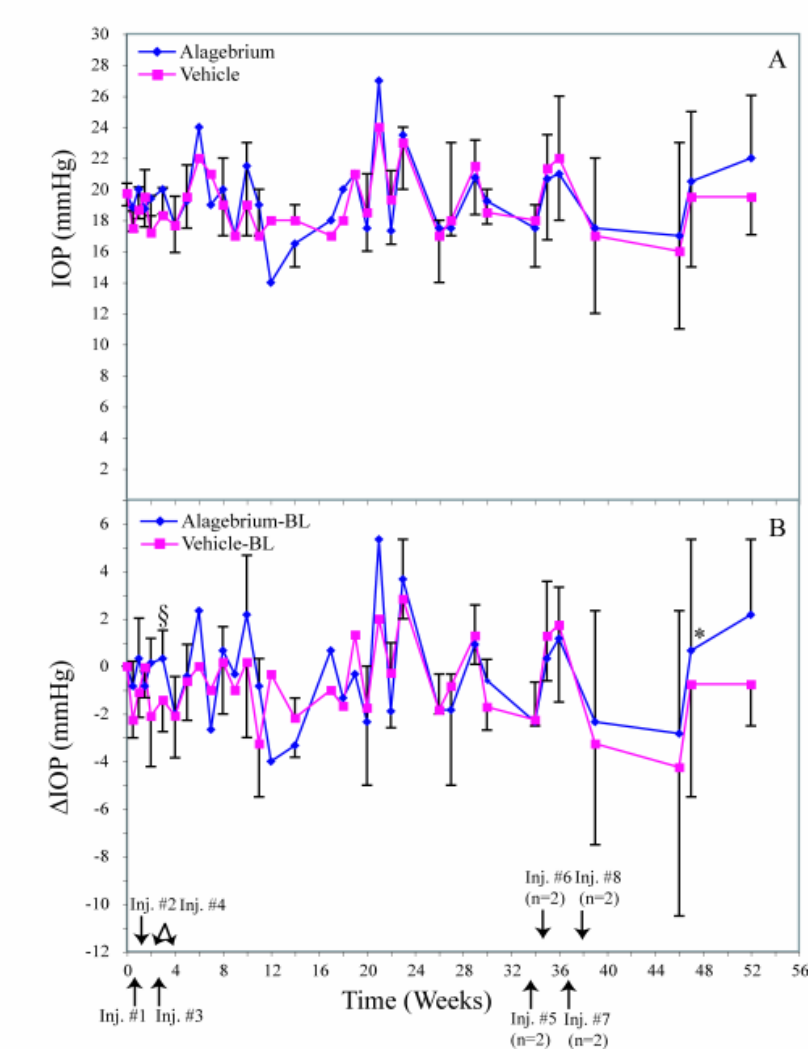

FIGURE 1

Intraocular pressure (IOP) after alagebrium. Mean IOP (mm Hg) (top) and IOP difference (bottom) in alagebrium-treated and vehicle-treated eyes prior to and up to 52 weeks postinjection. Data are mean±SEM for number of monkeys each contributing one alagebrium-treated and one vehicle-treated eye. IOP was measured prior to the first alagebrium or vehicle treatment, prior to each injection, after the fourth (final) injection, and prior to accommodation and outflow facility measurements. Arrows on the figure indicate injection times. Since IOP measurements were taken prior to other testing, IOPs were not taken in all 6 animals at exactly the same times; thus the number of monkeys contributing data for each time point differs in some cases and is as follows: n=6 (baseline, 0.5, 1, 3, and 4 weeks), n=5 (2 weeks), n=4 (1.5 and 5 weeks), n=3 (22 and 35 weeks), n=2 (8-11, 14, 20, 22-34, 36-52 weeks), n=1 (6, 7, 17-19 weeks). Significantly different from the contralateral eye corrected for preinjection baseline: §P<.01; *P<.05.

REFRACTION AND ACCOMMODATION

Refractions prior to any injections were no different between the eyes to be treated with alagebrium and those to be treated with vehicle (Table 2). Following injections, there was a tendency toward greater myopia in alagebrium-injected eyes than in vehicle-injected eyes, which was significant only at the 6-month time point. No significant differences were found in maximal accommodative response to intramuscular pilocarpine or intravenous pilocarpine at any time point when comparing alagebrium-treated vs control eyes or when comparing alagebrium-treated and control eye accommodative responses to their respective preinjection maximal accommodative responses (Figure 2).

TABLE 2. BASELINE REFRACTION PRIOR TO AND AFTER INJECTIONS WITH ALT-711 OR VEHICLE*

Time	REFRACTION (DIOPTERS)		REFRACTION DIFFERENCE			
	Alag	Veh	Alag - Veh	Alag - BL	Veh - BL	(Alag - BL) – (Veh - BL)
BL pre-trt (n=6)	-0.75 ± 1.06	-0.96 ± 0.52	-0.37 ± 0.75			
1-2 wk post-trt (n=5)	-1.79 ± 1.33	-0.23 ± 0.54	-0.43 ± 0.88	0.70 ± 0.50	0.77 ± 0.32	-0.07 ± 0.44
2 mo post-trt (n=6)	-2.04 ± 1.21	-0.73 ± 0.29	-1.06 ± 1.13	-0.47 ± 0.63	0.23 ± 0.49	-0.69 ± 0.65
4 mo post-trt (n=6)	-1.71 ± 1.28	-1.17 ± 0.92	-0.88 ± 0.78	-0.72 ± 0.71	-0.21 ± 0.68	-0.51 ± 0.89
6 mo post-trt (n=6)	-1.75 ± 0.87	-0.32 ± 0.67	-1.39 ± 0.99	-0.38 ± 0.48	0.64 ± 0.64	**-1.03 ± 0.34†**
BL 2 wk post-2nd trt (8 mo post-1st trt) (n=2)	-1.75 ± 0.25	-1.50 ± 0.5	-0.25 ± 0.75	-0.75 ± 0.75	-0.25 ± 0.50	-0.50 ± 0.25
BL 2 mo post-2nd trt (10 mo post-1st trt) (n=2)	-1.15 ± 0.65	-0.75 ± 0.15	-0.40 ± 0.80	-0.15 ± 1.15	0.50 ± 0.85	-0.65 ± 0.30

Alag, alagebrium; BL, baseline; inj, injection; trt, treatment; Veh, vehicle.
*Data are mean ± SEM for number of monkeys each contributing one alagebrium-treated and one vehicle-treated eye. Data correspond to time points for intramuscular pilocarpine testing in Figure 2A.
†Significantly different from 0.0 by the two-tailed paired *t* test: $P \leq .05$.

OUTFLOW FACILITY

Baseline outflow facility (Table 3) was no different between alagebrium-treated and vehicle-treated eyes at any time point before injection or after injection. Outflow facility response to intravenous pilocarpine was significantly higher (37.0 ± 6.0%; $P < .005$) at 6 months in alagebrium-treated eyes compared to controls when corrected for their respective 6-month post-treatment baseline outflow facility, but not at any other time points before or after alagebrium or vehicle treatment. In the 2 monkeys treated with a second round of alagebrium or vehicle injections, outflow facility response to intravenous pilocarpine was not significantly different 2 weeks or 2 months later. As expected,[34] post-pilocarpine outflow facility was significantly higher in both treated and control eyes when compared to the corresponding ipsilateral baseline outflow facilities at nearly every time point.

SLIT-LAMP BIOMICROSCOPY

Eyes were free of biomicroscopic cells and flare prior to each injection and prior to accommodation and outflow facility experiments. Injections were spaced 3 to 4 days apart if possible. If any ocular inflammation was observed, the injections were postponed until the inflammation resolved. Accommodation and/or outflow facility measurements were not done at some time points (Table 1) for some monkeys because of inflammation of one or both eyes (3 outflow facility experiments), corneal cloudiness (2 accommodation experiments), and/or recurrent conjunctivitis (one outflow facility and one accommodation experiment).

MORPHOLOGY

The alagebrium-treated eyes in all 3 monkeys showed focal plaque formation in the juxtacanalicular meshwork/inner wall of Schlemm's canal with aggregates clumped in some, but not all, regions (Figure 3). The "plaques" consisted of fine fibrillar material and more homogeneous-appearing electron-light material adhering to and partly masking the fibrils. At many places the plaques were connected to the sheath of the elastic fibers, thus resembling the sheath-derived (SD) plaques found in eyes with POAG.[39,40] At some places fine fibrillar material had accumulated underneath the inner wall of Schlemm's canal. These accumulations were also more abundant in treated eyes than in untreated controls. In the remaining trabecular meshwork, the inner trabecular lamellae were partly destroyed and the trabecular cells contained numerous cisterns of rough endoplasmic reticulum.

AGE AND RAGE IMMUNOLABELING OF THE RETINA AND OPTIC NERVE HEAD

In vehicle-treated eyes, some immunolabeling for AGEs was detectable in the retinas (Figure 4). Retinal AGE immunolabeling mainly encompassed inner retinal layers and the inner limiting membrane. Most of the AGE immunolabeling in retina sections exhibited extracellular distribution. The optic nerve head also exhibited some extracellular immunolabeling for AGEs, which included laminar cribriform plates and nerve fibers. Alagebrium-treated eyes trended toward decreased AGE immunolabeling compared with the

corresponding vehicle-treated controls. However, this treatment effect was not prominently detectable in all areas examined. Immunolabeling of the optic nerve head for AGEs in alagebrium-treated eyes was relatively less than detected in vehicle-treated controls.

Vehicle-treated retina and optic nerve head tissues also exhibited some immunolabeling for RAGE (Figure 4). Based on the morphologic characteristics and retinal distributions of cell types, retinal RAGE immunolabeling mainly corresponded to retinal ganglion cells (RGCs), characterized by larger and lighter nuclei, and astrocytes, characterized by smaller and darker nuclei and close localization to retinal vasculature in the RGC layer. Similarly, the optic nerve head exhibited cellular immunolabeling for RAGE, in contrast to AGE immunolabeling, which was mostly extracellular. In alagebrium-treated eyes, parallel to decreased AGE immunolabeling, there was a slight increase detectable in RAGE immunolabeling of the retina and optic nerve head in some, but not all, slides obtained from alagebrium-treated eyes relative to their vehicle-treated controls.

No differences in anterior segment AGE and RAGE immunolabeling were detectable comparing alagebrium- and vehicle-injected eyes.

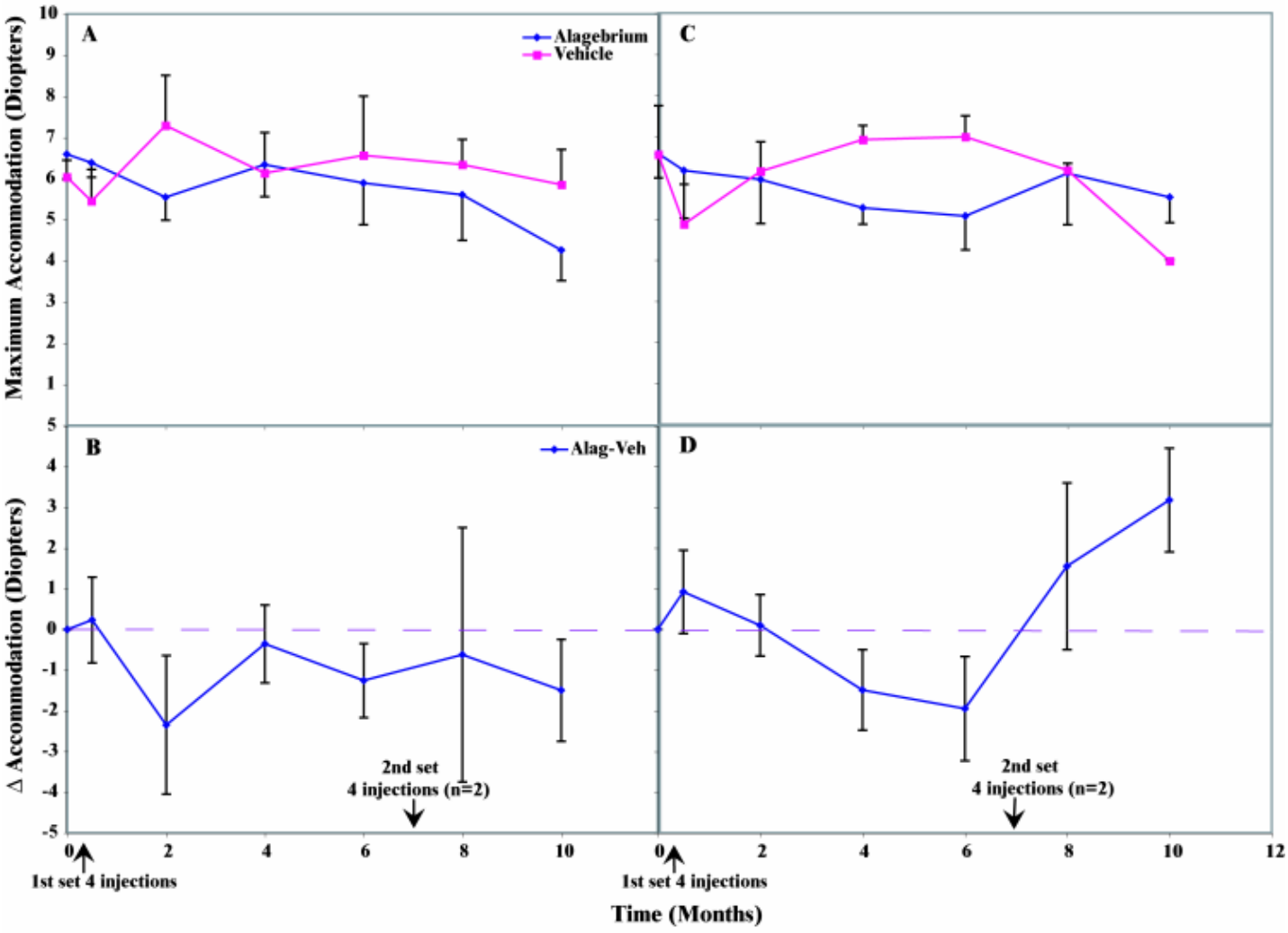

FIGURE 2

Maximum accommodation after intramuscular or intravenous pilocarpine. Maximum (**A**) intramuscular or (**C**) intravenous pilocarpine-induced accommodative amplitude prior to and at 1 to 2 weeks and 2, 4, 6 months after the first set of injections with alagebrium or vehicle and 2 weeks and 2 months after the second set of injections with alagebrium or vehicle (8 and 10 months after the first set). Difference in (B) intramuscular or (D) intravenous pilocarpine-induced accommodative amplitude in alagebrium-treated vs vehicle-treated eyes at the same time points above. Data are mean±SEM for number of monkeys, after treatment with 1.5 mg/kg pilocarpine intramuscularly or intravenously, each contributing one alagebrium-treated and one vehicle-treated eye. A, B: n=6 except at 1 week (n=5) post-first set of injections and 2 weeks and 2 months post-second set of injections (n=2). C, D: Baseline and 6 months (n=6), 2 weeks and 2 months (n=5), 4 months (n=4), and 2 weeks and 2 months post-second set of injections (n=2).

TABLE 3. OUTFLOW FACILITY RESPONSE TO INTRAVENOUS PILOCARPINE AFTER ALAGEBRIUM OR VEHICLE*

TIME	OUTFLOW FACILITY (µL/min/mm Hg) ALAGEBRIUM	VEHICLE	ALAGEBRIUM/ VEHICLE
BL pre-trt (n=6)	0.40 ± 0.04	0.39 ± 0.05	1.14 ± 0.18
BL 2 wk post-trt (n=5)	0.29 ± 0.02	0.34 ± 0.02	0.88 ± 0.07
BL 2 mo post-trt (n=5)	0.32 ± 0.03	0.37 ± 0.04	0.89 ± 0.07
BL 4 mo post-trt (n=4)	0.44 ± 0.17	0.39 ± 0.05	1.18 ± 0.49
BL 6 mo post-trt (n=6)	0.29 ± 0.01	0.37 ± 0.04	0.83 ± 0.09
BL 2 wk post-2nd trt (8 mo post-1st trt (n=2)	0.37 ± 0.04	0.34 ± 0.03	1.12 ± 0.23
BL 2 mo post-2nd trt (10 mo post-1st trt (n=2)	0.35 ± 0.05	0.38 ± 0.03	0.95 ± 0.19
Pilo iv pre-trt (n=6)	1.08 ± 0.20	1.33 ± 0.31	1.14 ± 0.18
Pilo iv 2 wk post-trt (n=5)	0.56 ± 0.08	0.68 ± 0.07	0.85 ± 0.14
Pilo iv 2 mo post-trt (n=5)	0.86 ± 0.06	1.01 ± 0.18	0.94 ± 0.16
Pilo iv 4 mo post-trt (n=4)	0.77 ± 0.28	0.80 ± 0.16	0.95 ± 0.27
Pilo iv 6 mo post-trt (n=6)	0.84 ± 0.16	0.75 ± 0.11	1.13 ± 0.11
Pilo iv 2 wk post-2nd trt (8mo post-1st trt) (n=2)	0.78 ± 0.07	0.86 ± 0.21	0.95 ± 0.15
Pilo iv 2 mo post-2nd trt (10 mo post-1st trt) (n=2)	0.73 ± 0.22	0.51 ± 0.02	1.44 ± 0.47
Pilo iv/BL pre-trt (n=6)	2.68 ± 0.40†	3.49 ± 0.74‡	0.89 ± 0.14
Pilo iv/BL 2 wk post-trt (n=5)	1.96 ± 0.33§	2.01 ± 0.17¶	0.95 ± 0.11
Pilo iv/BL 2 mo post-trt (n=5)	2.70 ± 0.18¶	2.87 ± 0.65§	1.07 ± 0.14
Pilo iv/BL 4 mo post-trt (n=4)	1.90 ± 0.50	2.06 ± 0.40	0.91 ± 0.10
Pilo iv/BL 6 mo post-trt (n=6)	2.96 ± 0.53‡	2.11 ± 0.31‡	1.37 ± 0.06¶
Pilo iv/BL 2 wk post-2nd trt (8 mo post-1st trt) (n=2)	2.17 ± 0.44	2.50 ± 0.39	0.86 ± 0.04
Pilo iv/BL 2 mo post-2nd trt (8 mo post-1st trt) (n=2)	2.02 ± 0.34	1.35 ± 0.05	1.48 ± 0.19

BL, baseline; pilo, pilocarpine; trt, treatment
*Data are mean±SEM for number of monkeys each contributing one alagebrium-treated and one vehicle-treated eye.
Ratio significantly different from 1.0 by the two-tailed paired *t* test:
†$P \leq .01$.
‡$P \leq .02$.
§$P \leq .05$.
¶$P \leq .005$.

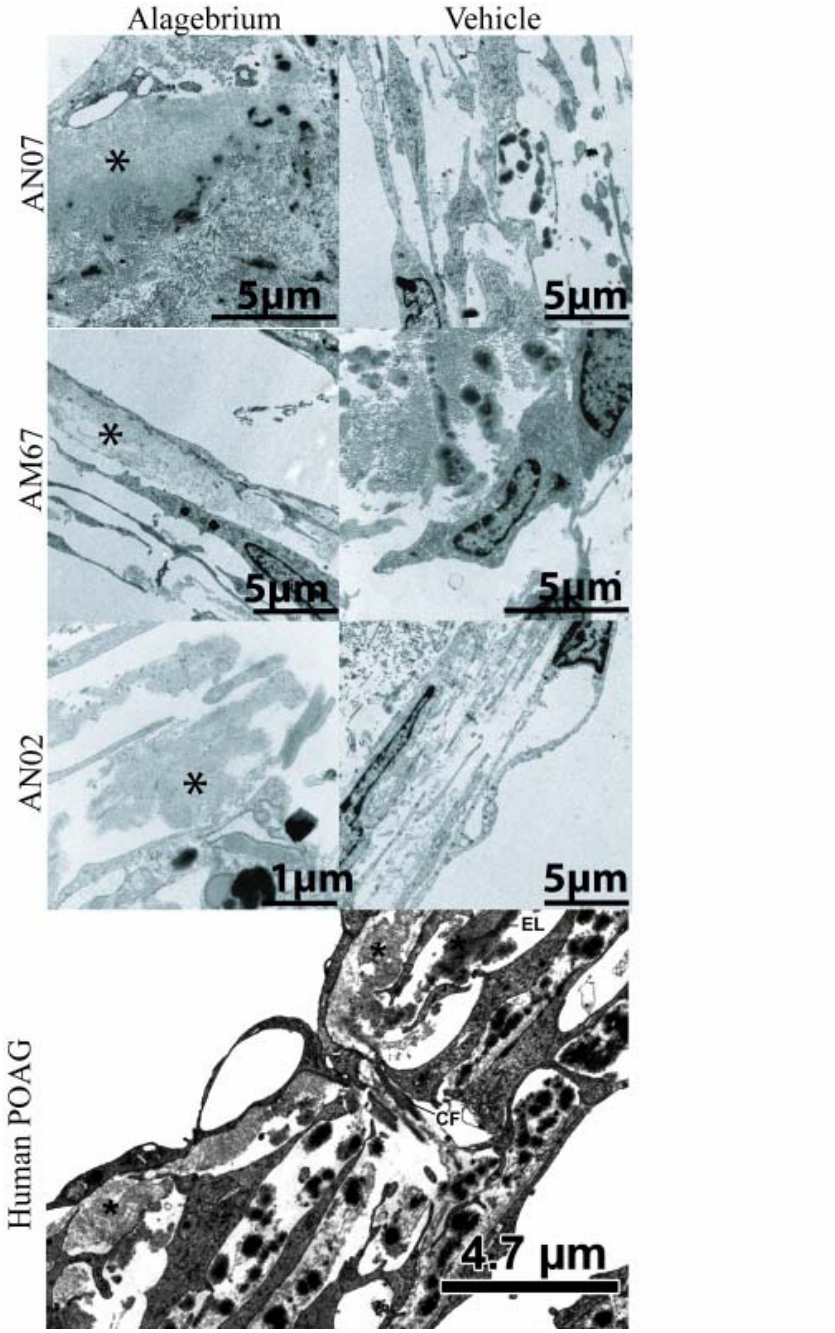

FIGURE 3

Juxtacanalicular meshwork/Schlemm's canal morphology after alagebrium. Electron micrographs of one or both eyes of 3 monkeys receiving alagebrium (approximately 1 mM final concentration) in one eye and vehicle to the opposite eye. AN02 and AN07 received a total of 8 injections; the second 4 injections were done ~6 months after the first 4 injections; both were euthanized ~3 months after the last injection. AM67 received a total of 4 injections and was euthanized ~6 months after the last injection. Plaques resembling those seen in human primary open-angle glaucoma (POAG) are indicated by the asterisks. For comparison, the bottom panel shows the inner wall of Schlemm's canal from a patient with POAG. Sheath-derived (SD) plaques (asterisk) underneath the inner wall endothelium are increased in these patients compared to age-matched controls. SD plaques are adherent to the elastic fibers (EL) and their connecting fibrils (CF).

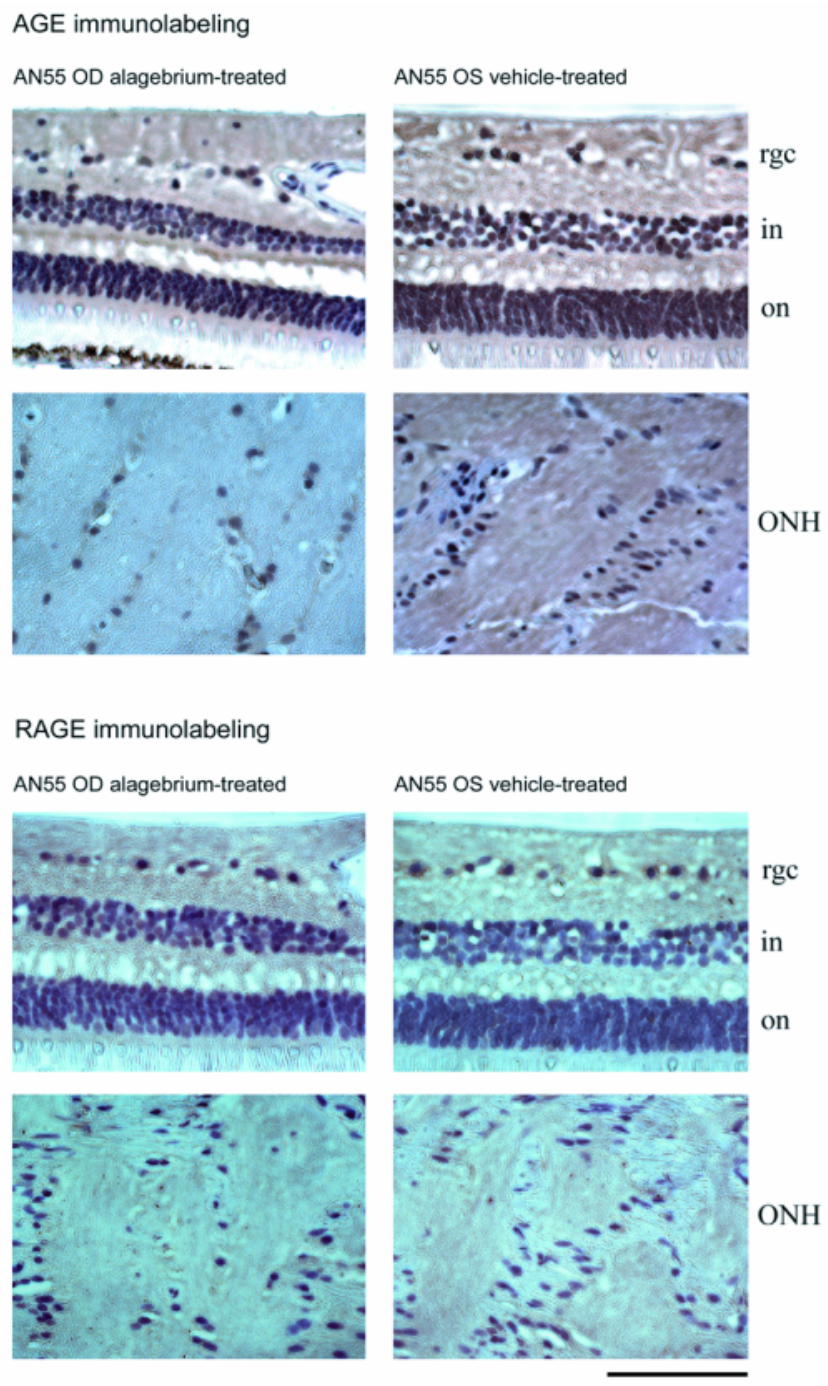

FIGURE 4

AGE and RAGE immunolabeling. Immunoperoxidase labeling of retina and optic nerve head sections for AGEs was decreased in alagebrium-treated eyes relative to corresponding vehicle-treated controls. Notice prominent extracellular AGE immunolabeling in the retinal ganglion cell layer in a vehicle-treated eye, while the alagebrium-treated eye of the same monkey exhibits less prominent immunolabeling for AGEs. Parallel to decreased AGE immunolabeling, there was a minor increase detectable in RAGE immunolabeling of the retina and optic nerve head in alagebrium-treated eyes relative to their vehicle-treated controls. RAGE immunolabeling mostly included retinal ganglion cells and glia in the inner retinal layers. rgc, retinal ganglion cell; in, inner nuclear layer; on, outer nuclear layer; ONH, optic nerve head. (Scale bar, 100 μm).

DISCUSSION

Intraocular injections of high concentrations of alagebrium, an AGE cross-link breaker, had no striking effects on IOP or refraction, accommodation, and outflow facility at baseline or in response to pilocarpine. We found an increase in outflow facility response to intravenous pilocarpine only at 6 months postinjection in alagebrium-treated compared to vehicle-treated eyes. Perhaps alagebrium sufficiently breaks the AGE cross-links to allow an enhancement of the outflow facility in response to pilocarpine, without increasing baseline outflow facility or IOP. However, the 6-month outflow facility measurement was performed in only 6 monkeys; there was no increase in outflow facility response to intravenous pilocarpine at 4 months after alagebrium injection or at 2 weeks or 2 months postinjection in the 2 monkeys that received a second set of 4 alagebrium injections. Also, there was no change in accommodative response to intravenous pilocarpine at any time before or after injection when comparing alagebrium-treated to vehicle-treated eyes.

Unexpectedly, morphologic examination of the juxtacanalicular/inner wall of Schlemm's canal region (the conventional outflow pathways) of nonhuman primates revealed what appeared to be increased plaque formation, similar to that seen only in human POAG.[39-41] Whether these plaques result from AGE fragments released after alagebrium treatment and then adhere to the inner-wall elastic fibrils is unknown. However, the regional distribution of plaques was insufficient to increase IOP or decrease baseline outflow facility in our monkeys.

Although no alterations in AGE or RAGE immunolabeling were detected in the anterior segment, a decrease in AGE immunolabeling of the retina and optic nerve head tissues was detectable in eyes treated with alagebrium compared with the vehicle-treated controls. The relative decrease in AGE immunolabeling of the retina and optic nerve head in alagebrium-treated eyes is of interest, as these oxidative stress-related reactive products have recently been associated with an age-dependent component of the neurodegenerative injury in glaucoma.[18] While intracellular aggregates of AGEs may interfere with normal cellular functions, including axonal transport, accumulation of AGEs in the extracellular matrix may decrease tissue elasticity with particular relevance to optic nerve head alterations in glaucoma.[42-44]

Enhanced accumulation of AGEs in laminar cribriform plates and blood vessels of the glaucomatous optic nerve head may increase the susceptibility of stressed axons to damage by compromising the axonal support function of the lamina cribrosa and/or impairing the microcirculation. The reduction in AGEs in eyes treated with AGE cross-link breaker might therefore be considered a beneficial effect. This immunohistochemical observation warrants further anatomic and functional studies to determine whether AGEs may be a worthwhile therapeutic target to reduce the rigidity of lamina cribrosa in glaucomatous eyes.

On the other hand, in addition to direct cytotoxic effects AGE aggregates, AGEs may initiate receptor-mediated signaling through RAGE binding. Parallel to decreased AGE immunolabeling in alagebrium-treated eyes, our immunohistochemical analysis also detected a minor increase in RAGE immunolabeling. The relative increase in RAGE, which may be an intrinsic response to decreased AGEs, could be considered an undesirable effect, since this multiligand signal transduction receptor can promote cell death and dysfunction and facilitate immune dysregulation.[9,45,46] It is possible that the AGE fragments released after alagebrium treatment may not be removed rapidly and may serve as ligands for RAGE. More important, multiple other ligands are also capable of activating RAGE signaling (reviewed in Barile and Schmidt[47]). This supports the likelihood of higher therapeutic usefulness of treatments antagonistic to RAGE interactions with its ligands. Whether pharmacologic applications targeting RAGE signaling may hold therapeutic promise for glaucoma remains to be determined.

In the present study, it appears that intraocular injection of AGE cross-link breakers is not likely to be an approach for POAG or presbyopia therapy but may actually generate a model for further study of glaucomatous-like plaque formation observed previously only in human POAG. For those studying alagebrium and/or other AGE cross-link breakers as potential therapy for diabetic complications, diabetic renal disease, cardiac dysfunction, or atherosclerosis, it may be appropriate to also look at any potential ocular effects of these compounds.

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PEER DISCUSSION

DR. MARK B. SHERWOOD: There are more than 60 papers in the literature on alagebrium, an advanced glycation end-products (AGE) crosslink breaker, but to the best of my knowledge this is the first study of the use of this type of agent in the eye. There is much documented evidence over the last 10 years that alagebrium is protective in cardiovascular injury related to diabetes, hypertension and aging, in diabetic nephropathy, in arterial aging and possibly osteoporosis associated with rheumatoid arthritis. The authors very cogently hypothesized that the eye, because of its high oxidative stress, and the known collagen increase plus decreased elastic compliance of certain tissues with ageing, might benefit from an AGE crosslink breaker like alagebrium.

The study results surprisingly did not show any major effect on intraocular pressure (IOP), outflow facility or accommodation in the aging monkey model. Why might this be? One possible reason is that a drug generally has to be maintained at a given level for a sufficient length of time to produce a significant effect. Since this is the first intraocular delivery of alagebrium the dosing must by nature be an educated guess based upon previous systemic studies and the practicalities of administration. Little and co-workers, in their study of 23 elderly patients with diastolic heart failure, used an oral dose of alagebrium of 420mg per day for 16 weeks and showed a decrease in left ventricular mass and improved left ventricular diastolic filling[1]. In the current study there was no increase in AGE or RAGE immunolabeling in the anterior segment while there was a decrease in AGE and a slight increase in RAGE noted in the retina and optic nerve head tissues. This may relate to a longer duration of effective drug levels when given intravitreally compared to intracamerally. Of interest, the last 2 monkeys in the study received a total of eight intravitreal and intracameral injections, but showed similar findings to the original group receiving four doses over 2 to 3 weeks. It is only feasible to give a limited number of intraocular injections, both in the research monkey model where sedation is required each time or in clinical practice where the patient needs to make an office visit. It might be important in future studies to see if oral administration can achieve adequate tissue levels in the eye and whether this might supplement tissue levels achieved by intraocular injections. Ocular examination of patients in current cardiovascular phase II and III studies with alagebrium may be helpful.

Another potential reason for the lack of demonstrated efficacy by alagebrium may be that only certain individuals with a specific disease co-morbidity or genetic make-up among a population group may respond, for example diabetics or elderly patients with POAG. Coughlan et al demonstrated that while infusion of AGEs to healthy rodents did induced renal cytosolic oxidative stress, unlike diabetic rats this did not lead to excess production of mitochondrial superoxide, because of the need for a sustained supply of glucose-derived NADH.[2] The 20 year old monkey approximates to a 50 or 60 year old healthy human.

Alagebrium has been noted to break only a subset of AGE crosslink structures (sugar-derived alpha-diketone bridges) which may limit effectiveness and the drug may have both a positive and negative effect on the end-points as demonstrated by the anterior chamber morphological findings.[3] One of the most intriguing findings of this research was the patchy plaque formation found in the inner, juxtacanalicular region of Schlemm's canal which appears very similar histologically to changes noted in human glaucoma. The clearance of the end-products of an AGE crosslink breaker may be different in the eye compared to the cardiovascular or renal systems and the authors point out the need to study this potential negative effect, together with the risks associated with the slight increase in RAGE immunolabeling in the retina and optic nerve head, further.

The authors are to be commended for their excellent pioneering work on this exciting new class of medication. Like Edison and his discovery of the electric light bulb, it may take numerous attempts to determine the best dose, delivery route and specific drug, but the potential for improving so many areas of common eye pathology is enormous. Due to their thorough approach the authors have also

sounded a warning bell about the possibility of longer-term deleterious ocular effects from this class of medication which may be important if its use becomes widespread in the management of cardiovascular disease.

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DR. ALLAN J. FLACH: I really enjoyed that also, Paul. Three questions come to mind. First, do you think you might have the potential for a model for open-angle glaucoma here? Secondly, have you analyzed the plaque histologically or chemically or with both methods, and compared it to the human plaques? Lastly, what about using carbachol in place of pilocarpine HCl? You would have both the direct and indirect effect. You have had the anti-cholinesterase effect and you would have the peculiar quality of carbachol to enhance the release of endogenous acetylcholine as with any other nerve stimulation, such as light.

DR. JAMES C. TSAI: First, in terms of financial disclosure, I serve as a consultant for several of the pharmaceutical companies that manufacture, market, and sell glaucoma therapeutic products. Paul, I was fascinated with your study because we and others have shown that intravitreal injections of erythropoietin (EPO) are neuroprotective in animal models of glaucoma. I recently reviewed a paper, which is likely to be published, that demonstrates EPO works primarily through its effects on the AGE and RAGE pathways. In thinking about potential other investigations with alagebrium, would be interested to learn if you looked at the posterior segment of the eye to determine whether these age link - cross link breakers could have some potential neuroprotective effects in glaucoma models. As I mentioned, our primary interest with erythropoietin is in trying to translate its basic science neuroprotective properties into clinically beneficial therapeutic options. However, the potential usefulness of EPO is limited because of possible angiogenic effects. If we could work on the RAGE and AGE pathways without causing undesired angiogenesis, then I believe it would be important to investigate this further.

DR. PAUL L. KAUFMAN: I thank you all for the very good comments. I especially thank Mark for the chance to discuss this over the last couple of days, essentially at the very last minute. We used, I will call them, single applications, but really intermittently, over a relatively short period of time. The goal was to break or disrupt complexes that already existed. In human trials one is thinking of a long-term experiment if one is trying to prevent or break up AGEs, or prevent AGEs from being laid down as one goes along; it is quite a different model. We actually did this after some discussions with the people at the company; however, it is a different company now that makes the alagebrium compound. To try and figure out the best way to do this was to answer a quick up or down question. If you hit the eyes with a pretty high dose, which was 4 injections over just a couple of week period of time to both the anterior and posterior segment, could you see a dramatic change in any of the parameters that we were investigating? I completely agree with Mark that it is not really a model for treating or preventing chronic disease; it is really more of an acute structure/function experiment with all of the pros and cons that kind of model can have.

With respect to Allan Flach's questions, and again thank you Allan, for always coming up with good questions, we do not know whether it is a model for POAG. Because the amount of plaque formation that we saw was distributed so unevenly around the circumference, and assuming the rest of the circumference was normal, we would not expect intraocular pressure to go up in these animals. Had we done it longer, and more intensively, or had we given it orally as Mark suggested, maybe it could have generated an increased intraocular pressure and thus a model, but certainly there was no suggestion that doing it the way we did would produce a model. I cannot answer your question for other modes of administration. As to the plaques themselves, they were not analyzed biochemically, but were looked at morphologically in the way the group in Erlangen looks at these findings. Dr. Lütjen-Drecoll told me that they were the closest things she had ever seen to what we see in POAG in the human and that she was astonished when she saw them. Obviously, she knows the difference between a monkey and a human eye, but she was wondering what had been done to these animals. Having said that, our interpretation is based on structural and not biochemical criteria. With respect to why we used pilocarpine instead of carbachol, again, the idea was to induce accommodation quickly. As you know from our papers, we do use both drugs and everything you said about the difference between them is correct. We did not believe that it was particularly important to do that in this instance. Remember that we gave this drug systemically and one of the differences between pilocarpine and carbachol is that pilocarpine is only a partial agonist and it is a relatively lousy agonist for the cardiovascular and GI system compared to carbachol. There is sort of an ocular selectivity with pilocarpine and you can stabilize the cardiac and GI systems against the effects of pilocarpine with a very tiny dose of atropine that does not affect the eye. Actually, Göran Tornqvist worked this out about 40 years ago and the doses are well known. Systemic administration allowed us to deliver the same dose to both eyes and we tend to use pilocarpine for that purpose. Certainly with corneal iontophoresis we want to stimulate the hell out of the system so we use carbachol for sure.

Jim, that is a great comment. Whether this is a better way or cleaner way to look at neuroprotection, I do not know. I will remind the group that Gülgün Tezel had a paper about two years ago in *IOVS* in which she studied age-matched glaucomatous and normal

human eyes and found that increased accumulation of AGE products in the retina and the optic nerve, not primarily in RCGs. This was a function of age, but interestingly the findings were even more pronounced in glaucomatous eyes. Whether breaking them up would be neuroprotective or not was the unanswered question in her study of course, and that is one of the reasons we performed this study. We did not do a neuroprotection study, but one could imagine doing that. It would be nice to have a model in the primate to study that question and maybe one could try to do that in the laser model of glaucoma until something better comes along. I thank everybody for their attention and for their questions. I hope I have answered them reasonably and satisfactorily.